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PROVISIONAL APPLICATION COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(b)(2).

ATTORNEY DOCKET NO: 21001.004

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TITLE OF THE INVENTION: **SELECTIVE GROWTH MEDIUM FOR *LISTERIA SPP.***

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ENCLOSED ARE THE FOLLOWING DOCUMENTS:

[X] Specification consisting of eight (8) pages
[X] A check for $80 to cover the provisional filing fee
[X] The Commissioner is hereby authorized to charge any fees or credit any overpayments to Deposit Account Number 18-2055.
[ ] Other (specify)_____

The invention was NOT made by an agency of the United States Government or under a contract with an agency of the United States Government.

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PROVISIONAL PATENT APPLICATION

SELECTIVE GROWTH MEDIUM FOR *LISTERIA SPP.*

BACKGROUND OF THE INVENTION

Considerable microbiological research has been devoted to understanding the nutritional requirements and environmental conditions that promote selective growth of *Listeria spp.* Dependable selective culturing of *Listeria spp.* is becoming increasingly important in the food industry because of evolving federal and state regulations requiring more frequent monitoring of food-processing equipment and environments. *Listeria spp.* is considered to be a critical indicator of the effectiveness of industrial sanitation practices for two principle reasons: 1) organisms of the genus *Listeria* are ubiquitous; and 2) the species *Listeria monocytogenes* is pathogenic and thus a cause of concern for public health officials.

A number of putative media for selective culture of Listeria spp. have been described in the literature. For example, see Lovett, J.D. et al. (1987) "*Listeria monocytogenes* in raw milk: detection, incidence, and pathogenicity," *J. Food Prot.* 50:188-192. This paper describes an enrichment broth for selective isolation of *Listeria spp.*

See also, Fraser, J and W. Sprerber (1988) "Rapid detection of *Listeria* in food and environmental samples by Esculin hydrolysis," *J. Food Prot.* 51:726-765. Fraser and Sprerber developed a selective medium that exploits the high salt tolerance of *Listeria spp.*, and its ability to hydrolyze esculin. Esculin is a glucoside (6-(beta-D-glucopyranosyloxy)-7-hydroxy-2H-1-Benzopyran-2-one, CAS No: 531-75-9) obtained from *Æsculus hippocastanum* (the horse chestnut) and is characterized by its fine blue fluorescent solutions. In this approach, the beta-glucosidase activity of *Listeria* hydrolyzes esculin. The hydrolysis products, in combination with iron salts present in the media, yield a black pigment that is used as a colorimetric indicator of a positive sample.

Donnelly & Baigent developed a modified medium similar to the Fraser & Sprerber broth but lacking the colorimetric indicator. This Donnelly & Baigent medium exploits the salt tolerance of *Listeria spp.* in conjunction with several antibiotics to yield a medium

selective for the growth of *Listeria*. See Donnelly, C.W. & G.J. Baigent (1986) "Method for flow cytometirc detection of *Listeria monocytogenes* in milk," *Appl. Environ. Microbiol.* 52:689-695.

All of the aforementioned selective media have an inherent disadvantage in that they slow the overall growth rate of *Listeria* cells to achieve inhibition of competitive micro-flora in the sample being tested. Furthermore, the combination of high salt concentration and antibiotics prevents the growth of certain strains of *Listeria*, most notably *L. ivanovi* and *L. grayi*. Another complicating aspect of the conventional selective media is the presence of acriflavin. Acriflavin is an acridine dye that is an effective inhibitor of competitive gram-positive bacteria such as *Bacillus spp.* Unfortunately, acriflavin not only is a suspected carcinogen but is also a fluorphore that is incorporated into the DNA and proteins of growing cells. Thus the acriflavin causes unwanted fluorescent interference in many fluorescence-based assays such as enzyme-linked immunosorbent assays (ELISA) and the polymerase chain reaction (PCR). Many commercially available *Listeria* detection products rely upon the use of fluorescent reagents for analyte detection.

There remains in the field a long-felt and unmet need for a *Listeria*-selective medium: 1) that does not appreciably interfere with the growth rate of *Listeria spp.*; 2) that does not yield bacterial biomass contaminated with interfering fluorophores; and 3) that strongly inhibits the growth of non-*Listeria* organisms.

DETAILED DESCRIPTION OF THE INVENTION

The need for a selective medium that does not compromise the growth of *Listeria* was a primary motivation for development of the novel medium disclosed herein. At the outset, it was observed that high salinity was necessary criterion to control certain bacterial competitors, such as *Enterococcus spp.* and *Bacillus spp.* In the present medium, however, the lithium chloride concentration is greatly reduced as compared to conventional media, and acriflavin is not used. The present medium also contains the antibiotic compound nitrofurantoin, which is effective to inhibit the growth of competing gram-postive bacteria. Nitrofurantoin is non-

fluorescent, and thus does not interfere with ELISA- or PCR-based detection protocols. Moreover, nitrofurantoin has significant anti-microbial activity against many potential gram positive competitors, at concentrations from about one to two orders of magnitude lower than the minimum inhibitory concentration for *Listeria spp.* See Soriano, F. et al. (1995) "Antimicrobial susceptibilities of *Corynebacterium* species and other non-spore forming gram-positive bacilli to 18 antimicrobial agents," *Antimicrob. Agents Chemother* 39:208-214; and Safdar, A. & D. Armstrong (2003) "Antimicrobial activities against 84 *Listeria monocytogenes* isolates from patients with systemic Listeriosis at a comprehensive cancer center (1955-1997)," *J. Clin. Microbiol.* 41:483-485.

EXAMPLES

The following Examples illustrate the features of the novel selective medium disclosed and claimed herein. The Examples are included solely to provide a more complete disclosure of the invention and do not limit the scope of the medium disclosed and claimed herein in any fashion.

Example 1:

TABLE 1: Medium Formulation, Versions PDX-1 and PDX-2.

Ingredient	PDX-1 (g/L)	PDX-2 (g/L)
Tryptone	17.0	17.0
Peptone	3.0	3.0
Sodium Chloride	5.0	5.0
Dibasic Potassium Phosphate (anhydrous)	6.0	6.0
Yeast extract	6.0	6.0
Cyclohexaimde	0.05	0.05
Acriflavin	0.01	-
Naladixic acid	0.04	0.04
Esculin	1.0	1.0

The solid ingredients were dissolved in distilled water and autoclaved at 121 psig for 15 min to sterilize. After cooling, the following supplements were added:

TABLE 2: Supplements.

Supplement name	PDX-1	PDX-2
Ceftazimide	40 mg/L	40 mg/L
Phosphomyocin	40 mg/L	40 mg/L
Polymyxin E (Colistin)	10 mg/L	10 mg/L
Ferric Ammonium Citrate	0.5 g/L	0.5 g/L
Lithium Chloride*	5.0 g/L	5.0 g/L
Nitrofurantoin**	-	6 mg/L

*Lithium chloride is exothermic when dissolved in water. Appropriate care must be taken when adding it to the medium.

**Nitrofurantoin is insoluble in water. A 10 mg/mL stock solution was made in sterile DMSO. The nitrofurantoin/DMSO stock solution was then added to the rest of the medium (600 µl of stock solution/L medium yields 6 mg/L nitrofurantoin in the final medium). Solid-medium plates were made from the liquid media by adding 15 g agar per liter of liquid medium, bringing the media to a boil to dissolve the agar, cooling the solutions, and sterilizing the same.

Example 2: Comparison of Growth Rates: PDX-1 vs. Fraser Broth:

The purpose of this Example is to compare the growth rate of L. monocytogenes in Fraser broth versus the growth of L. monocytogenes in PDX-1 liquid medium.

Cultures of L. monocytogenes (100 µl of 10-7 dilution; 1/10 serial dilutions on peptone from overnight L. monocytogenes culture in tryptone soy broth [TSB]) were added to 3 mL of Fraser broth and 3 mL of PDX-1. Every hour starting at the time of inoculation, 100 µl of both the PDX-1 medium and Fraser medium were plated on PALCAM plates in duplicate and incubated at 32 °C for the enumeration of colonies. (For data on PALCAM plates, see Van Netten et al. (1989) "Liquid and solid selective differential media for the detection and enumeration of Listeria monocytogenes," Int. Food Microbiol. 8:299-316. PALCAM plates are available commercially from a number of international suppliers.)

TABLE 3: Data (Expressed as CFU/0.1 mL).

Hour	0	1	2	3	4	5
PDX-1	65	73	69	72	131	273
	67	72	76	100	149	257
Fraser	59	74	66	68	94	70
	57	66	81	81	77	100

The data show that the *Listeria* in PDX-1 sample were able to recover from inoculation and start growth faster than the samples grown in Fraser broth. Also of interest is the fact that both sets of samples were inoculated from the same stock and had the same volume of inoculation. Thus the difference in initial cell counts between the two media (65 and 67 for PDX-1; 59 and 57 for Fraser broth) is significant, suggesting that the PDX-1 medium is less stressful to the cells at initial inoculation. In both runs, the *Listeria* displayed greater survivability in the PDX-1 medium as compared to the Fraser broth..

Example 3: Growth of ATCC Cultures on Solid PDX-1, PDX-2, and Modified Oxford Broth:

The purpose of this Example was to plate out ATCC cultures of various bacteria, including *Listeria spp.* on solid PDX-1 and PDX-2 media to obtain a record of their respective colony morphologies, as well as to compare these morphologies to those of corresponding colonies grown on conventional media.

A loop of overnight TSB culture was streaked out on PDX-1, PDX-2, and Oxford broth supplemented with moxalactam. The various primary cultures were obtained from the American Type Culture Collection, Manassas, Virginia. Plates were stored at 37°C and checked at 20 and 40 hours. The results are shown in Table 4:

TABLE 4: Growth of Different Species on Various Solid media After 20 Hour Incubation at 37°C.

Species	PDX-1	PDX-2	Oxford + Mox
S. choleraesuis	-	-	-
M. luteus	-	-	-
S. aureus	-	-	Regular, round, off-white colonies
L. welshimeri	+	+	+
L. ivanovii	+	+	+
L. grayi	-	-	Area of some discoloration, without any noticeable colonies where the streak started
L. seelgreri	-	-	-
L. monocytogenes	+	+	+
L. innocua	+	+	+
E. faecalis	Discoloration without visible colonies at location of start of streak	Discoloration without visible colonies at location of start of streak	Discoloration without visible colonies at location of start of streak

As can be seen from the data, the media according to the present invention are highly selective for the growth of *Listeria spp.* and highly inhibitory of the growth of non-Listeria species.

CLAIMS

What is claimed is:

1. A *Listeria spp.*-selective medium comprising, in combination, nitrofurantoin and no greater than about 0.01 g/L acriflavin.

2. A *Listeria spp.*-selective medium comprising nitrofurantoin and which is devoid of acriflavin.

3. The medium of Claim 2, further comprising lithium chloride.

4. The medium of Claim 3, further comprising esculin.

5. A *Listeria spp.*-selective medium comprising, in combination, tryptone peptone, sodium chloride, dibasic potassium phosphate, yeast extract, cyclohexaimde, acriflavin, naladixic acid, esculin, ceftazimide, phosphomyocin, polymyxin E, ferric ammonium citrate, lithium chloride, and nitrofurantoin.

6. A *Listeria spp.*-selective medium comprising, in combination,

tryptone	17.0 g/L
peptone	3.0 g/L
sodium Chloride	5.0 g/L
dibasic potassium posphate (anhydrous)	6.0 g/L
yast extract	6.0 g/L
cclohexaimde	0.05 g/L
nladixic acid	0.04 g/L
eculin	1.0 g/L
ceftazimide	40 mg/L
phosphomyocin	40 mg/L
polymyxin E (Colistin)	10 mg/L
ferric ammonium citrate	0.5 g/L
lithium chloride	5.0 g/L
nitrofurantoin	6 mg/L.

ABSTRACT

Disclosed is a growth medium containing nitrofurantoin. The growth medium is selective for the growth of *Listeria spp.*